

Evaluation of Culture Media for Detecting Airborne *Salmonella enteritidis* Collected with an Electrostatic Sampling Device from the Environment of Experimentally Infected Laying Hens

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ABSTRACT Detection of *Salmonella enteritidis* in the environment of commercial laying hens is critical for reducing the production of contaminated eggs by infected flocks. In the present study, an inexpensive and portable electrostatic air sampling device was used to collect *S. enteritidis* in rooms containing experimentally infected laying hens. After hens were orally inoculated with a phage type 13a *S. enteritidis* strain and housed in individual cages, air samples were collected 3 times each week with electrostatic devices onto plates of 6 types of culture media (brilliant green agar, modified lysine iron agar, modified semisolid Rappaport-Vassiliadis agar, Rambach agar, XLD agar, and XLT4 agar). Air sampling plates were incubated at 37°C, examined visually for presumptive identification of typical *S. enteritidis* colonies and then subjected to confirmatory enrichment culturing. Air sam-

ples (collected using all 6 culture media) were positive for *S. enteritidis* for 3 wk postinoculation. Because visual determination of the presence or absence of typical *S. enteritidis* colonies on air sampling plates was not consistently confirmed by enrichment culturing, the postenrichment results were used for comparing sampling strategies. The frequency of positive air sampling results using brilliant green agar (66.7% overall) was significantly greater than was obtained using most other media. A combination of several plating media (brilliant green agar, modified lysine iron agar, and XLT4 agar) allowed detection of airborne *S. enteritidis* at an overall frequency of 83.3% over the 3 wk of sampling. When used with appropriate culture media, electrostatic collection of airborne *S. enteritidis* can provide a sensitive alternative to traditional methods for detecting this pathogen in the environment of laying flocks.

(Key words: air, chicken, egg, electrostatic sampling device, *Salmonella enteritidis*)

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INTRODUCTION

The transmission of *Salmonella enteritidis* infection to humans by internally contaminated table eggs presents a continuing challenge to public health on several continents despite nearly 2 decades of sustained efforts to control the problem (Angulo and Swerdlow, 1999; Centers for Disease Control, 2003). Because infected hens can deposit this pathogen into the edible contents of developing eggs (Gast and Beard, 1990; Gast and Holt, 2000; Gast et al., 2002), detection of *S. enteritidis* in laying flocks is vital to prevention of the transmission of disease to consumers (Hogue et al., 1997). Bacteriologic culturing of environmental samples from laying houses is the most commonly used method for detecting *S. enteritidis* in commercial flocks (White et al., 1997; President's Council on

Food Safety, 1999). The presence of *S. enteritidis* in the environment of laying flocks is generally accepted as a sensitive and relevant indication that contaminated eggs might be produced (Henzler et al., 1998; Garber et al., 2003). However, the collection and processing of environmental samples collected by the standard drag-swab technique (applied to surfaces such as floor litter, manure pits, or walkways) can be labor-intensive, time-consuming, and sometimes hazardous. Accordingly, alternative methods for efficiently detecting *S. enteritidis* in poultry house environments would be highly useful in programs that aim to reduce the risk of egg-transmitted human disease.

The detection of airborne bacteria is a testing option that could provide information about the presence of pathogens in poultry houses based on a relatively small number of easily collected samples. Air circulation often plays a major role in the perpetuation of *Salmonella* con-

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Abbreviation Key: BG = brilliant green agar; MLI = modified lysine iron agar; MSRV = modified semisolid Rappaport-Vassiliadis agar; PI = postinoculation; RAM = Rambach agar.

tamination in poultry house environments and in the transmission of infection within flocks. The lengthy environmental persistence of *S. enteritidis* on poultry farms (Davies and Breslin, 2003a; Gradel and Rattenborg, 2003) can provide an opportunity for extensive airborne dissemination of the pathogen via contaminated dust and aerosols. Airborne horizontal transmission of *S. enteritidis* infection has been reported for both chicks and laying hens in diverse types of housing (Lever and Williams, 1996; Nakamura et al., 1997; Gast et al., 1998; Holt et al., 1998). This airborne circulation of bacteria may also create an opportunity for efficient detection of the presence of *S. enteritidis* in poultry houses. Various bacteria (including *Salmonella*) have been detected in air samples collected in poultry hatcheries, houses, and processing facilities (Bailey et al., 1996; Hoover et al., 1997; Whyte et al., 2001). Airborne salmonellae in poultry environments have been detected by sampling air filters from ventilation systems, by passive exposure of agar plates, and by devices that actively draw air onto media for sampling (Berrang et al., 1995; Hoover et al., 1997; Kwon et al., 2000a,b). However, most commercially available air sampling devices are too expensive for routine use.

By reducing dust levels in poultry hatcheries and housing facilities, electrostatic space chargers (negative air ionizers) have been reported to bring about corresponding reductions in the levels of airborne bacteria (including salmonellae) and to diminish the experimental transmission of *Salmonella* to chicks (Gast et al., 1999; Holt et al., 1999; Mitchell et al., 2002; Mitchell and Waltman, 2003; Richardson et al., 2003). The ability of electrostatic space chargers to attract and hold bacteria associated with airborne particulate matter has also been recently adapted to develop a technique for detecting the presence of airborne pathogens. In a prior study, a novel electrostatic sampling device was used to detect airborne *S. enteritidis* in the environment of infected laying hens. This device detected *S. enteritidis* significantly more often did than passive exposure of agar plates and at a similar frequency to that of a far more expensive impaction sampler (Gast et al., 2004). However, that study did not assess any possible differences in the effectiveness of the electrostatic air sampling device when used with different culture media. The objective of the present study was to compare 6 types of agar culture media used in an electrostatic sampling device for detecting the presence of airborne *S. enteritidis* in a room containing experimentally infected laying hens.

MATERIALS AND METHODS

Experimental Infection of Laying Hens

In each of 2 replicate trials, 40 laying hens from a specific-pathogen-free flock of Single Comb White Leghorn

chickens were housed individually in laying cages in a disease-containment facility. The hens (34 to 40 wk old at the beginning of each trial) were distributed evenly throughout 2 tiers of cages located on both sides of an isolation room and were provided with water and pelleted feed ad libitum. The floor area of this room was 33.1 m², and the air was replaced approximately 12 times/h (similar to the ventilation rate at mild temperatures in commercial poultry houses). The floor was not cleaned to remove accumulated waste and debris (including manure, feathers, and dust) during each trial. One week after placement in the isolation room, all hens were inoculated orally with a phage type 13a isolate of *Salmonella enteritidis* prepared by overnight incubation at 37°C in tryptone soya broth² and dilution in 0.85% saline to yield approximately 3.4×10^9 cfu of *S. enteritidis* per 1.0-mL dose.

Fecal Samples

Fecal samples from each hen were collected and cultured for the presence of *S. enteritidis* immediately before inoculation and at 1, 2, and 3 wk postinoculation (PI). Sterile cotton swabs were used to remove freshly voided feces from food-grade plastic-foam trays placed beneath each cage for enrichment culturing by previously described methods (Gast et al., 1993).

Air Samples

To test for the presence of airborne *S. enteritidis* in the room containing infected laying hens, plates of agar media were exposed using an experimental electrostatic sampling device that was developed and produced at our laboratory (Gast et al., 2004). The electrostatic sampling device is simple (no moving parts), compact (20 cm long \times 14 cm wide \times 15 cm high and weighing 0.9 kg), battery-operated, and inexpensive (\$50 for parts per device). All electronic components except for the plug-in batteries are housed in a waterproof enclosure that allows disinfection by spraying. This device functions by applying a strong electrostatic field to attract charged airborne dust particles and aerosols (and associated microorganisms) onto the surface of agar media plates.

Air samples were collected by distributing 6 electrostatic devices at evenly spaced intervals on top of the upper tier of cages around the isolation room. The electrostatic sampling devices were used to collect air samples onto plates of 6 different agar culture media: brilliant green agar (BG)³ supplemented with 0.02 mg of novobiocin⁴/L, modified lysine iron agar (MLI),² modified semi-solid Rappaport-Vassiliadis agar (MSRV),² Rambach agar (RAM),⁵ XLD agar³ supplemented with 0.02 mg of novobiocin/L, and XLT4 agar.⁴ One plate of each medium was exposed during every sampling interval, and the various media were rotated among the 6 different sampling locations around the room throughout the experiment. Air samples were collected by exposure of plates for 1 and 2 h, 3 times weekly, for a total of 6 samples on each medium

²Oxoid Ltd., Basingstoke, Hampshire, UK.

³Becton, Dickinson, and Co., Sparks, MD.

⁴Sigma Chemical Co., St. Louis, MO.

⁵E. Merck, Darmstadt, Germany.

⁶GraphPad Software, San Diego, CA.

per week. Air samples were collected before inoculation of the hens with *S. enteritidis* and for 3 wk PI.

After exposure during sampling, the agar plates were incubated for 40 h at 37°C. Typical *Salmonella* colonies on each agar plate after incubation were determined by visual inspection. To confirm the presumptive visual identification of *S. enteritidis* colonies, the agar was then removed from each plate and transferred to 50 mL of Rappaport-Vassiliadis broth.² After incubation of the broth cultures for 24 h at 42°C, a portion from each sample was streaked onto plates of BG agar and incubated for 24 h at 37°C. The identities of prospective colonies of *S. enteritidis* on these plates were confirmed by standard biochemical and serological methods (Waltman et al., 1998). No other *Salmonella* serotypes were detected in this study.

Statistical Analysis

For each replicate trial, significant differences ($P < 0.05$) between sampling exposure intervals (1 and 2 h) in the frequency of recovery of airborne *S. enteritidis* were determined by applying Fisher's exact test to summary data for all sampling wks combined, organized into 2×2 contingency tables. Significant differences between agar culture media in the frequency of recovery of airborne *S. enteritidis* with the electrostatic sampling device were determined by applying Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test to summary data for all sampling wks and exposure intervals combined. Data were analyzed with Instat biostatistics software.⁶ Because the statistical relationships between treatment groups were similar for the 2 trials, the results were combined for presentation.

RESULTS

No fecal samples collected before oral inoculation of the hens were positive for *Salmonella*, but 98.8% of such samples were positive for *S. enteritidis* at 1 wk PI. The frequency of fecal shedding of *S. enteritidis* then declined steadily to 63.8% at 2 wk PI and to 40.0% at 3 wk PI.

Determination of the presence or absence of *S. enteritidis* colonies by visual inspection of exposed air sampling plates after the initial period of incubation was not consistently supported by subsequent enrichment culturing of the agar media (Figure 1a). For all media and all sampling dates combined, only 52.1% of visually *S. enteritidis*-positive plates were eventually confirmed by enrichment culturing. Likewise, only 70.1% of all visually *S. enteritidis*-negative plates were confirmed by enrichment culturing. Accordingly, because visual identification of *S. enteritidis* colonies on exposed agar plates was not dependable, enrichment culturing results were used for comparing different media in this study.

Different intervals of exposure (1 and 2 h) of agar media plates to air from the room that contained the infected hens had no significant influence on the frequency of recovery of *S. enteritidis* (Figure 1b). For all media and all

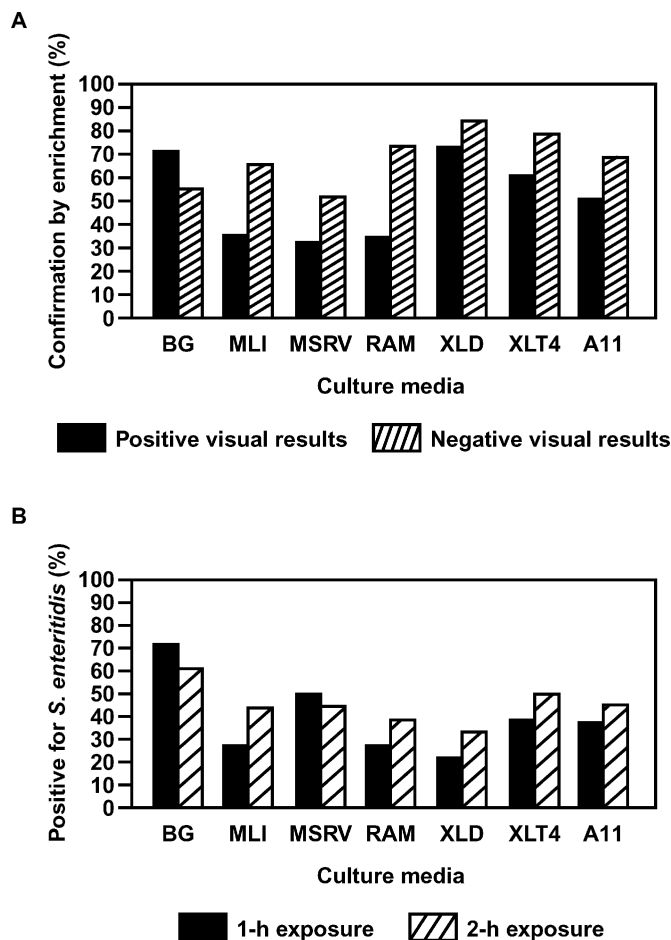


FIGURE 1. Evaluation of culture methods and media for detecting airborne *Salmonella enteritidis* collected with an electrostatic sampling device from the environment of experimentally infected laying hens. In each of 2 trials, 40 orally inoculated laying hens were housed in a single room and 18 air samples were collected over a 3-wk period onto each of 6 media: brilliant green (BG), modified lysine iron (MLI), modified semisolid Rappaport-Vassiliadis (MSRV), Rambach (RAM), XLD, and XLT4 agar. (a) Confirmation of visual identification of *S. enteritidis* colonies on agar media by subsequent enrichment culturing, presented as the percentage of visually positive or negative plates exposed in the electrostatic sampling device that yielded the same results after enrichment. (b) Frequency of detection of *S. enteritidis* by enrichment culturing after exposure of agar plates in the electrostatic sampling device for 1 or 2 h.

sampling dates combined, 37.0% of plates exposed for 1 h and 45.1% of plates exposed for 2 h were positive for *S. enteritidis* after enrichment.

For all media and sampling dates combined, 41.2% of air sampling plates were positive for *S. enteritidis* (Table 1). The overall frequency of positive results using BG (66.7%) was greater ($P < 0.05$) than the corresponding frequencies for XLD (27.7%), RAM (33.3%), MLI (36.1%), or MSRV (38.9%) but not for XLT4 (44.4%). No other significant differences among media were evident. At 1 wk PI, the frequency of *S. enteritidis*-positive air samples ranged from 41.7% (with MLI or XLD) to 83.3% (with BG). By 3 wk PI, the frequency of positive results ranged from 16.7% (with RAM) to 66.7% (with BG).

The frequency of detection of airborne *S. enteritidis* that was achieved using any single agar medium could be

TABLE 1. Evaluation of agar culture media for detecting airborne *Salmonella enteritidis* collected with an electrostatic sampling device from the environment of experimentally infected laying hens¹

Culture media	Air samples positive for <i>S. enteritidis</i> / total (%)			
	1 wk PI ²	2 wk PI	3 wk PI	All weeks
Brilliant green	10/12 (83.3)	6/12 (50.0)	8/12 (66.7)	24/36 (66.7) ^a
Modified lysine iron	5/12 (41.7)	4/12 (33.3)	4/12 (33.3)	13/36 (36.1) ^{bc}
Modified semisolid Rappaport-Vassiliadis	6/12 (50.0)	2/12 (16.7)	7/12 (58.3)	14/36 (38.9) ^{bc}
Rambach	6/12 (50.0)	4/12 (33.3)	2/12 (16.7)	12/36 (33.3) ^{bc}
XLD	5/12 (41.7)	2/12 (16.7)	3/12 (25.0)	10/36 (27.7) ^{bc}
XLT4	9/12 (75.0)	4/12 (33.3)	3/12 (25.0)	16/36 (44.4) ^c
All media	40/72 (55.6)	22/72 (30.6)	27/72 (37.5)	89/216 (41.2)

^{a,b}Means followed by different superscripts are significantly different ($P < 0.05$).

¹In each of 2 trials, 40 orally inoculated laying hens were housed in a single room.

²Postinfection.

increased by using certain combinations of media. The best recovery of *S. enteritidis* with a set of 3 media was attained using MLI, XLT4, and BG. If the results obtained using these media (in separate electrostatic sampling devices at the same time) were combined so that a positive recovery of *S. enteritidis* on any of the 3 plates was considered to be a positive result for the overall test, then *S. enteritidis* was detected in all 12 air sample tests (for all sampling days and exposure intervals combined) at 1 wk PI and in 11 of 12 tests (91.7%) at 3 wk PI.

DISCUSSION

A strong positive correlation has previously been established between the presence of *S. enteritidis* in the house environment of commercial laying flocks and the production of contaminated eggs (Hogue et al., 1997; Henzler et al., 1998). Because *S. enteritidis* can persist in laying houses for many months (Davies and Breslin, 2003a,b), environmental testing is a highly relevant surveillance tool for identifying flocks that might pose a risk to public health (and which thus merit further investigation). Dust particles and aerosols are extensively and continuously generated in poultry houses and may play significant roles in spreading and perpetuating *Salmonella* and other bacteria as air circulates throughout buildings. The potential for air circulation to disseminate pathogens in poultry houses is illustrated by several prior studies that reported the airborne transmission of *S. enteritidis* infection to chickens (Lever and Williams, 1996; Nakamura et al., 1997; Gast et al., 1998; Holt et al., 1998). Airborne salmonellae have been isolated from turkey rearing houses (Hoover et al., 1997) and broiler chicken hatching cabinets (Berrang et al., 1995; Bailey et al., 1996). Nevertheless, air samples have not been widely used for detecting *S. enteritidis* in poultry environments, perhaps because commercially available air samplers are generally quite expensive.

The electrostatic air sampling device used in the present study is inexpensive, portable, and simple to operate. It was developed after earlier research demonstrated that reducing airborne dust levels in poultry facilities by electrostatic attraction led to reduced levels of airborne bacteria (Holt et al., 1999; Mitchell et al., 2002; Mitchell and Waltman, 2003). In an initial study, this electrostatic sam-

pling device performed comparably to a much more expensive impaction sampler for detecting *S. enteritidis* in rooms containing infected chickens (Gast et al., 2004). In the present investigation, the electrostatic sampler (using any of 6 agar culture media) was able to detect airborne *S. enteritidis* in the environment of laying hens for 3 wk after oral inoculation. The floor of the isolation room that housed the infected chickens was not cleaned during the course of this experiment to allow dust, manure, and feathers to accumulate as they would under commercial conditions. The introduction of *S. enteritidis* into this environment by shedding in the feces of infected hens evidently resulted in persistent environmental contamination with the pathogen, including continuing circulation in the air. Even as the incidence of ongoing fecal shedding of *S. enteritidis* from hens declined substantially by the third week PI, the environmental contamination load was apparently sufficient to permit the detection of airborne *S. enteritidis* at a high frequency. In both the present investigation and a prior study (Gast et al., 2004), *S. enteritidis* was detected at similar frequencies in fecal samples and air samples from rooms containing experimentally infected hens. In commercial poultry facilities, parameters including airflow volume and direction, dust level, relative humidity, and bird stocking density would affect the requirements for the number and location of air samples necessary to efficiently detect *S. enteritidis*.

Visual identification of *S. enteritidis* colonies on incubated air sampling plates in the present study was not dependably accurate. Although further selective enrichment of the agar media increased the duration of the testing process by several days, the resulting improvement in the accuracy of the results was ample justification for the added time. When the lengthier, enrichment-based protocol is used with the electrostatic air sampling device, the efficacy of an agar culture medium for recovering *S. enteritidis* is determined entirely by its ability to support *Salmonella* growth after exposure to air (without regard to its differential properties). As an alternative to enrichment culture, the electrostatic collection of bacteria using a solid or liquid medium might be combined with a rapid assay (such as PCR or ELISA) to detect specific airborne pathogens.

A large and diverse assortment of culture methods and media are used in different laboratories to test poultry samples for *Salmonella* (Waltman and Mallinson, 1995). Previous investigations have reached different conclusions regarding the optimal media for recovering salmonellae from particular types of samples, so no single medium can be identified as ideal for all purposes (Miller et al., 1991; Waltman et al., 1992; Cox and Berrang, 2000). Although all 6 tested media were used successfully to detect airborne *S. enteritidis* with the electrostatic sampling device in the current study, BG agar provided the highest frequency of positive results. Collecting samples onto more than one type of medium improved the recovery of airborne *S. enteritidis* in comparison to single media and thus represented a more promising strategy for effective air sampling with the electrostatic device.

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